



How to grow your cable bacteria: Establishment of a stable single-strain culture in sediment and proposal of *Candidatus Electronema aureum* GS

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ABSTRACT

Cable bacteria are multicellular filamentous bacteria within the Desulfobulbaceae that couple the oxidation of sulfide to the reduction of oxygen over centimeter distances via long distance electron transport (LDET). So far, none of the freshwater or marine cable bacteria species have been isolated into pure culture. Here we describe a method for establishing a stable single-strain cable bacterium culture in partially sterilized sediment. By repeated transfers of a single cable bacterium filament from freshwater pond sediment into autoclaved sediment, we obtained strain GS, identified by its 16S rRNA gene as a member of *Ca. Electronema*. This strain was further propagated by transferring sediment clumps, and has now been stable within its semi-natural microbial community for several years. Its metagenome-assembled genome was 93% complete, had a size of 2.76 Mbp, and a DNA G + C content of 52%. Average Nucleotide Identity (ANI) and Average Amino Acid Identity (AAI) suggest the affiliation of strain GS to *Ca. Electronema* as a novel species. Cell size, number of outer ridges, and detection of LDET in the GS culture are likewise consistent with *Ca. Electronema*. Based on these combined features, we therefore describe strain GS as a new cable bacterium species of the candidate genus *Electronema*, for which we propose the name *Candidatus Electronema aureum* sp.nov. Although not a pure culture, this stable single-strain culture will be useful for physiological and omics-based studies; similar approaches with single-cell or single-filament transfers into natural medium may also aid the characterization of other difficult-to-culture microbes.

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Introduction

The vast majority of microbial organisms has yet to be cultured; it has been estimated that only 1 % of microbes can be cultured with standard isolation techniques [35]. A more recent meta-analysis came to a similar estimate and furthermore pointed out that >80% of all microbial cells belonged to yet uncultured genera [17]. Although molecular techniques enable identification of microbes directly from the environment, their exact physiological characterization and full taxonomic classification requires cultivation [36]. A primary obstacle for cultivation in pure culture is the failure to mimic natural growth conditions; an alternative to

isolation is thus continuous cultivation of environmental samples. Such measures have led to the recovery of difficult-to-culture bacteria, allowing them to grow and be studied in a laboratory. Prominent examples are the enrichment of anammox bacteria [37], the cocultivation of the members of the ANME consortia [1], and the decade-long cultivation of a Lokiarchaeota strain [12]. While persistent maintenance of difficult-to-culture microbes is a Sisyphean task, it is only by cultivation that such thorough characterization becomes possible.

The cable bacteria are yet another difficult-to-culture group of bacteria that inhabit aquatic sediments. These filamentous bacteria conduct electrons in a gradient system of spatially separated oxic and sulfidic zones [28]. The individual filaments are centimeters long and composed of thousands of cells with a shared periplasmic space enclosing conductive fibers, used for long distance electron transport [13,22]. Despite many attempts, none of the known cable

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bacteria species can be grown in pure culture. They have therefore been classified directly from their habitat by 16S rRNA gene amplicon sequencing and visualized by fluorescence *in situ* hybridization (FISH) and transmission and scanning electron microscopy (TEM/SEM) [28]. Such environmental samples are high in cable bacteria diversity and contain several species [9,21]. A taxonomic study of this diversity suggests that they form a monophyletic clade in the Desulfobulbaceae family split into two genera, the marine *Candidatus* Electrothrix and the freshwater *Candidatus* Electronema [38]. The above-mentioned methods, while useful for initial classification, are imperfect for continued studies due to major restrictions. First, the approaches are destructive and preclude any further analyses of the cable bacteria filaments. Additionally, the high interspecies diversity complicates the reconstruction of high quality genomes, previously circumvented by single filament whole genome amplification, resulting in fragmented genomes [14].

Here we present a simple two-step process to establish a single strain cable bacterium culture: First, we remove a single cable bacterium filament from an environmental sample and second, we reintroduce the same filament into autoclaved sediment from its source environment. The autoclaved source sediment functions as a suitable medium replacement with favorable geochemical conditions to stimulate growth. This process reduces the diversity of the cable bacteria population to a single strain, facilitating the assembly of a high-quality cable bacteria genome.

Materials and methods

Sediment sampling and pre-incubation

Sediment from a freshwater pond in Aarhus, Denmark (56.164796, 10.207805) was collected with a shovel on the 28th

of January 2014. The presence of cable bacteria in the sediment was verified by microscopy (see below). The sediment was blended with a kitchen hand blender, sieved (approx. 1 mm) and homogenized. The prepared sediment was packed in glass liner cores (100 mm × 20 mm) and kept in a closed plastic box. Cable bacteria developed in the top centimeter of the sediment within a month, and were ready to be used as a source for single filament transfers.

Preparing sediment for inoculation

Sediment from the same pond was blended, sieved, homogenized as described above, and funneled into 2-liter blue cap bottles. The bottles were autoclaved at 120 °C for 30 min. The autoclaved sediment was transferred from the bottles directly into 50 ml falcon tubes sealed with parafilm. The cores were placed in a closed plastic box, where the sediment was allowed to settle overnight prior to inoculation. This procedure was repeated approximately each month as needed for cable bacteria transfers.

Additional tests were done with sequential autoclaving sediment in 50 ml falcon tubes. The tubes were autoclaved, left to settle for 1 day, and then re-autoclaved. These double-autoclaved falcon tubes were directly inoculated.

Filament picking and inoculation

Small glass hooks for isolating filaments (tip diameter ~10–20 µm) were constructed by heating a Pasteur pipette and pulling the tip into an L-shape [32]. Using a dissection microscope, a small clump of cable bacteria-rich sediment from the prepared enrichment core was placed on one end of a microscope slide and a small clump of autoclaved sediment was placed at the opposite end of the slide, approx. 5 cm away (Fig. 1a). 1 ml of filter sterilized tap

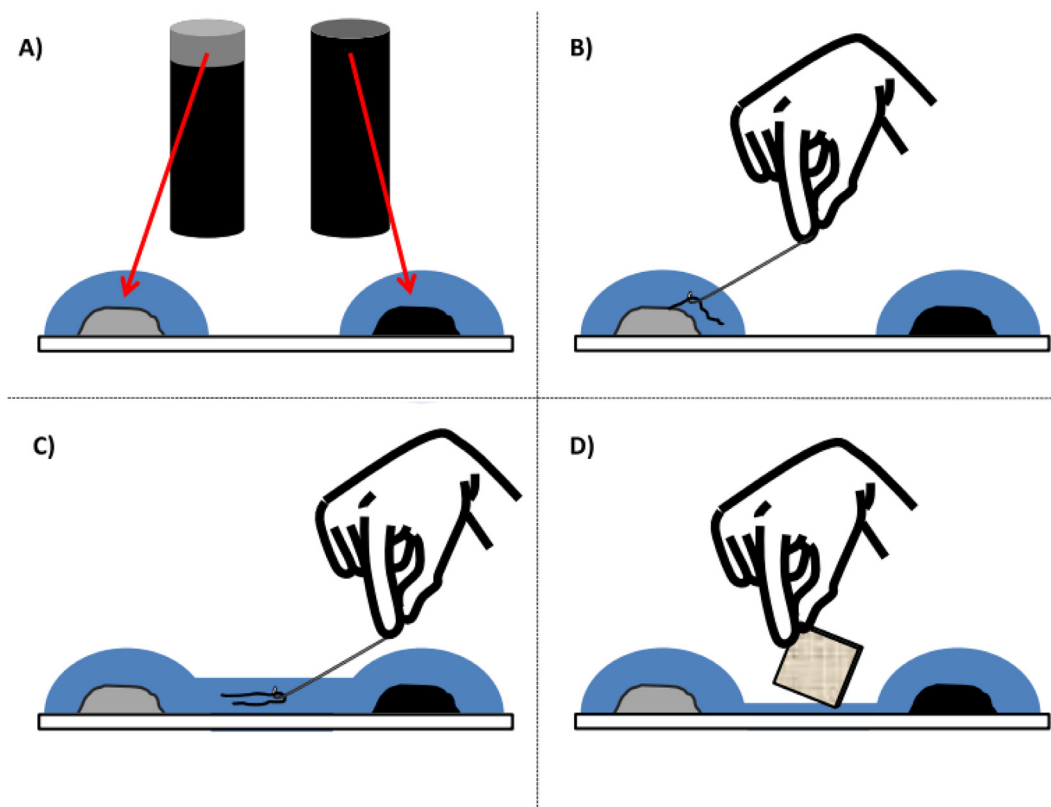


Fig. 1. Method for inoculating sediment with a single cable bacterium filament. A) Sediment with cable bacteria and autoclaved sediment is placed on opposite ends of a microscope slide. B) A single cable bacterium filament is picked with a glass hook. C) The filament is dragged through a sterile water bridge towards the autoclaved sediment. D) Excess water is removed with a paper towel.

water was added on top of each clump, creating a droplet of clear water around the sediment. Using a sterile glass hook, a single cable bacterium filament was pulled from the sediment and into the water phase, where it was cleaned of any visible attached sediment by dragging it against the glass surface (Fig. 1b). A small corridor of filter-sterilized water was added between the two clumps of sediment. The single cable bacterium filament was swiftly dragged by glass hook through the corridor and into the clump of sediment from the autoclaved core (Fig. 1c). The corridor of water was immediately dried with a paper towel to prevent the migration of other microbes to the sterile sediment (Fig. 1d). The freshly inoculated clump of autoclaved sediment was returned to an autoclaved sediment core and mixed into the top of the sediment by stirring. This procedure was done in 10 replicates. The 10 inoculated cores were covered with Parafilm to prevent evaporation, incubated for 3 weeks at 15 °C, and then checked microscopically for growth of cable bacteria. Twelve 50 ml falcon tubes with double autoclaved sediment were inoculated in the same manner. Uninoculated, autoclaved cores were used as negative controls.

Establishment and maintenance of the cable bacteria culture

Of the initial 10 replicates, one single core with the fastest cable bacteria growth (labelled strain GS) was chosen as inoculum for further single filament transfers. Transferring single cable bacterium filaments (~20 replicates) to freshly autoclaved cores was henceforth routinely repeated every month for a total of 11 single filament transfers. In parallel to the single filament transfers, autoclaved cores were inoculated with a pea sized clump of inoculum sediment. This simpler method of inoculation replaced the single final transfers after the 11th transfer, and this method has been ongoing for 7 years.

Microscopic analysis

After 3 weeks, samples from all inoculated and negative control cores were inspected for cable bacteria by phase contrast microscopy (Axio Observer, Zeiss, Germany). For microscopic analysis, sediment was put into a microscope slide with a trench in the center as previously described [2,3]. Using this approach, cable bacteria move out from the sediment in this “trench slide” towards the oxic/anoxic interphase at the edge of the slide, making them easily visible by phase-contrast microscopy.

The identity of cable bacteria was verified by fluorescence *in situ* hybridization (FISH) for selected samples. To prepare the samples for FISH, trench slides were left upside down in a closed box at 20 °C for 2 days to allow the filamentous bacteria to stick to the slides' cover glass. The cover glass was then removed and dried at 15 °C, dehydrated by gently flooding with ethanol (50, 75, and 96% ethanol for 3 min each), and finally dried at 46 °C. FISH using probes DSB706 [19] and EUB338 I–III [8], labeled with Cy3 and fluorescein, respectively, was performed according to previously published protocols [27] with 35% v/v formamide in the hybridization buffer and a hybridization temperature of 46 °C for 1.5 h. All washing steps were done by gently flooding the cover glass to prevent removal of the attached bacteria.

The width of the filaments in the single strain cable bacterium culture was assessed in triplicate over a growth period of 32 days. Approximately 200 cable bacteria filaments were measured at each timepoint. Measurements were performed using phase contrast at 400× magnification, using the distance tool and theoretical scaling in Zen Black edition (Zeiss, Germany 2016).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) of the single strain cable bacterium culture was done as previously described [38].

Amplicon sequencing

The relative abundance of cable bacteria in the single-strain culture was assessed by 16S rRNA gene amplicon sequencing. In brief, DNA was extracted from two sediment cores 35 days after a cable bacteria transfer using the DNeasy PowerLyzer PowerSoil Kit (Qiagen). The V3–V4 region was PCR amplified using the primers Bac341F and Bac805R [11] and sequenced on an Illumina MiSeq instrument. The resulting sequences were analyzed using the DADA2 pipeline [5]; for details see Scholz et al. [33].

Microsensor measurements

After the seventh round of single filament transfers, profiling of pH and electric potential (EP) using microsensors was performed in the sediment cores as previously described [10,23]. Cores inoculated with single filaments, with a clump of sediment, or non-inoculated control cores were profiled daily from day 9 to 19 of the incubation. All microprofiles were recorded from 0.1 cm above the sediment surface to 1.0 cm depth. pH profiles were run with a 100 µm step size, wait time of 6.0 s and 2.0 s measure period. EP profiles were run with a 100 µm step size, wait time of 0.5 s and 1.0 s measure period. All profiles were conducted in triplicates.

Metagenome sequencing and genome assembly

Cable bacteria genomes were assembled from metagenomes constructed at two different time points. After 9 single filament transfers, sterilized sand was sequentially added on top of three sediment cores for 7 days to a final height of 5 mm, forcing the cable bacteria to migrate into the sand layer towards the oxic surface (Fig. S1A). The resulting cable bacteria-enriched sand layer was harvested. After 11 transfers, two additional cores were sampled. From one core, filaments were directly collected with a glass hook into sterile water (Fig. S1B). From the other core, sediment was added into a trench slide and the cable bacteria that moved out into the glass towards the oxic/anoxic interphase were scraped off with a sterile razorblade (Fig. S1C).

DNA was extracted from these five samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen). Metagenome libraries were prepared using the Nextera DNA Library Preparation Kit (Illumina) and sequenced using the Illumina MiSeq kit v3 with 2x300 bp reads. The reads were quality checked using FastQC v. 0.11.4 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed using Trimmomatic v. 0.33. [4]. A metagenome was assembled from the pooled reads of the five sequence libraries with IDBA v. 1.1.1 [26] and used to extract a cable bacterium genome as described in detail in Kjeldsen et al. 2019 [14].

Two metagenome libraries constructed after the 9th and 11th transfer were analyzed separately to compare the cable bacteria genomes by their average nucleotide identity (ANI). The reads of each library were mapped separately onto the previously assembled cable bacterium genome [14] with BBMap using a minimum identity threshold of 98%. The mapped reads were re-assembled using SPAdes v. 3.6.2 with settings: -careful -kmer 21,33,55,77,99,127.

Phylogenomic analysis

Single-copy phylogenetic marker genes were identified, aligned, and the alignments concatenated using the gtdbtk identify and gtdbtk align workflows from the Genome Taxonomy Database toolkit version 0.3.2 (GTDB-Tk) [6]. A whole-genome phylogenetic tree was constructed based on this alignment using MEGA-X version 10.0.5 [16] supported by 1000 bootstraps. The full length 16S rRNA gene sequences of the new isolate and 16 previously

published cable bacteria were aligned to the SILVA Release 119 SSU Ref database using the SINA aligner version 1.2.11 [29] and the alignment was manually inspected in ARB [18]. A phylogenetic tree was calculated by maximum likelihood and supported by 1000 bootstraps. 16S rRNA gene sequence similarities were calculated using the ARB neighbor-joining tool.

The average nucleotide identity (ANI) and average amino acid identity (AAI) between the single filament cable bacterium genome, *Ca. Electronema nielsenii* F1 and F5 and *Ca. Electronema palustre* F3 and F4 was calculated using both best hits and best reciprocal hits as described in Rodriguez-R [31].

Results and discussion

Growth of cable bacteria from single filament transfers

Microscopic observation of the source sediment in a trench slide showed high densities of cable bacteria that stretched out from the sediment towards the oxic/anoxic interface at the edge of the slide, marked by a clear veil of microaerophilic bacteria. In addition, eukaryotes, primarily ciliates and amoebae feeding on the microbial veil, and other filamentous bacteria were present (Fig. 2A). Cable bacteria and a microaerophilic veil were also

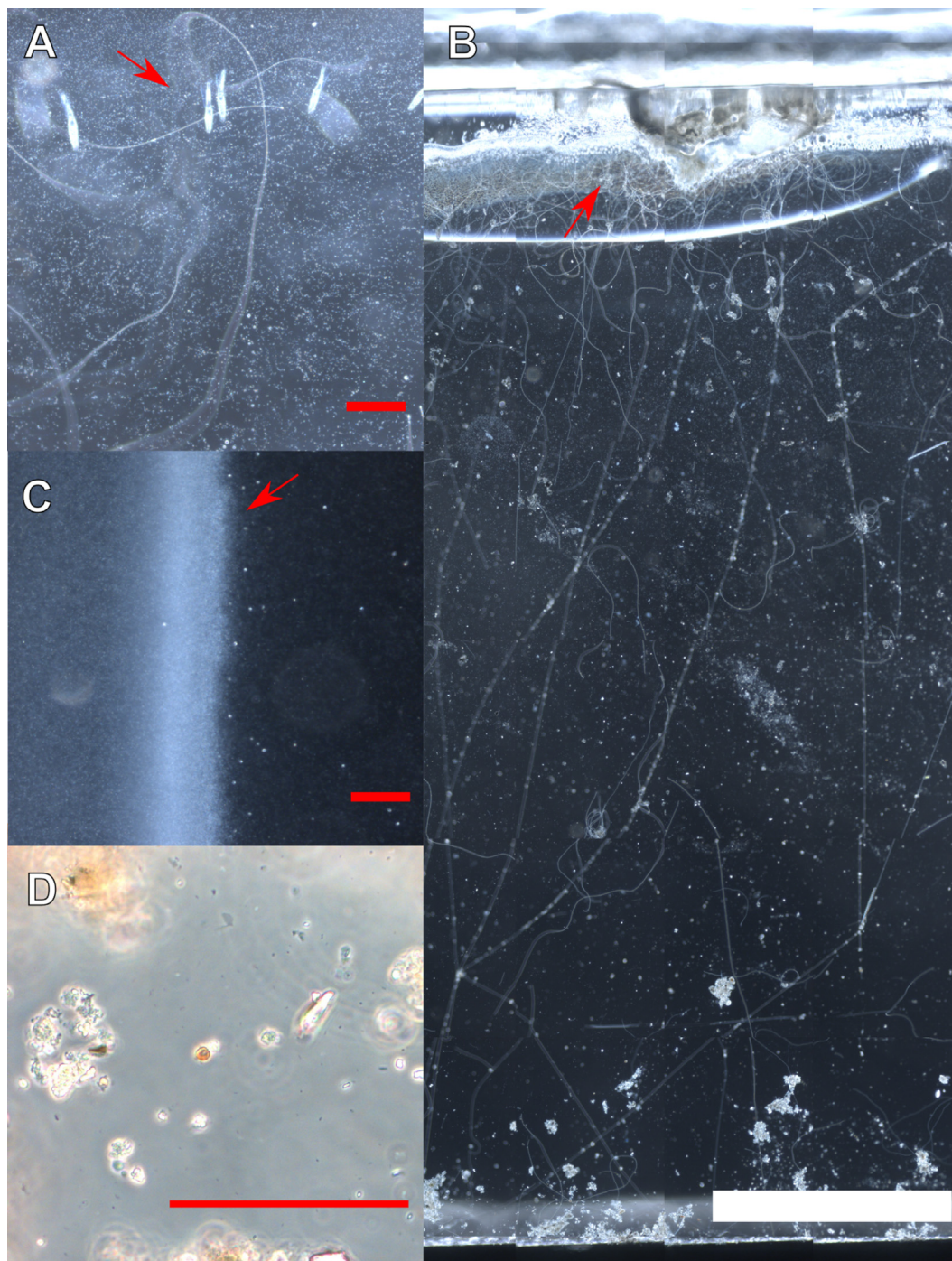


Fig. 2. Phase contrast micrographs of sediment incubations, white scale bar is 1 mm, red scale bars are 200 μ m. **(A)** Source sediment core with cable bacteria crossing the microaerophilic veil (red arrow), predation by multiple eukaryotes. **(B)** Autoclaved inoculated sediment with *Ca. Electronema aureum* GS from a single filament transfer core. The cable bacteria stretch from the sulfidic sediment (bottom) to the oxic water at the edge of the slide (red arrow). **(C)** Autoclaved, uninoculated sediment (negative control) with a clear microaerophilic veil (red arrow) but with no cable bacteria. **(D)** Double autoclaved sediment with no visible microbes.

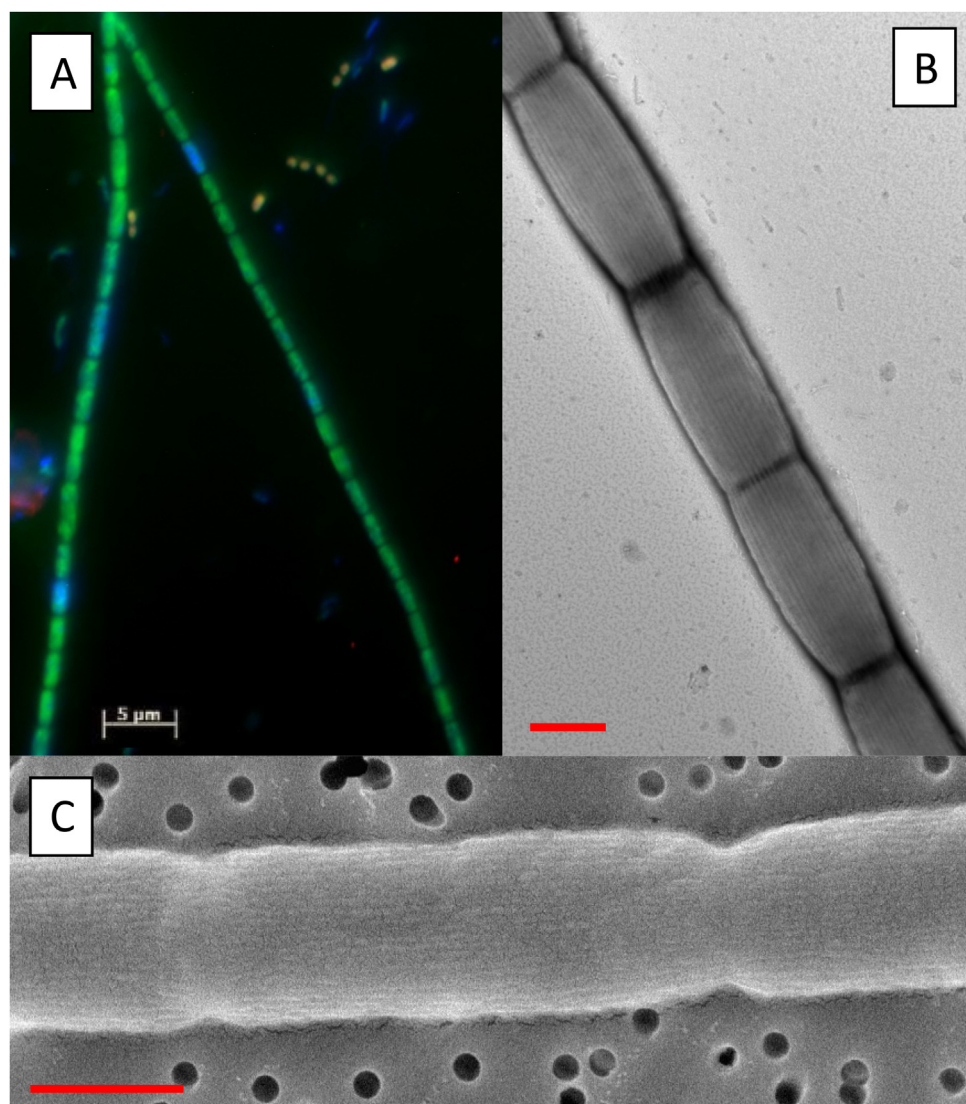


Fig. 3. Morphology of *Ca. Electronema aureum* GS. A, FISH with probes DSB706 (green) and EUB338 (orange) and stained with DAPI (blue); scale bar, 5 μm. B, Transmission Electron Microscopy (TEM) image of a single filament, showing the distinct longitudinal ridges. C, TEM image of a cross-section of a filament, showing the central core and surrounding layers.

observed in 3 out of the 10 initial single filament transfers, but here no eukaryotes or other filamentous bacteria were found (Fig. 2B). To our surprise, negative control cores (with autoclaved sediment, uninoculated) also showed a microaerophilic veil, yet no sign of any filamentous bacteria or eukaryotes (Fig. 2C). These observations show that it is possible to establish a cable bacterium culture from just a single filament and that our sediment autoclaving procedure was sufficient to kill large filamentous bacteria (incl. cable bacteria) and eukaryotes, but possibly too short to completely sterilize the 2 L batches of sediment.

In contrast, negative control cores with double-autoclaved sediment showed no growth at all, indicating that this treatment fully sterilized the sediment. Single filament transfers into this sterile sediment were unsuccessful at establishing a cable bacteria culture (Fig. 2D). Whether this was due to a strong alteration of the chemical composition of the sediment during autoclaving (summarized by Otte et al. [25]), or due to a lack of potentially essential microbial partners [39], remains an open question.

While single filament transfers were generally only successful in approx. 21% of attempts, inoculation with a pea sized clump of sediment worked much more consistently (close to 100%), and

was therefore chosen as the routine method of propagating the culture of strain GS after its initial establishment.

Identification and activity of cable bacterium strain GS

The filamentous bacteria observed by phase contrast microscopy were confirmed to be cable bacteria by FISH (Fig. 3A). The morphology of the filaments was likewise indicative of cable bacteria: TEM revealed that our culture had 40 ± 1 ($n = 22$) of the characteristic longitudinal ridges along the entire filament length (Fig. 3B), while SEM showed that the periplasmic ridges bridge the cell-cell junctions (Fig. 3C). The number and appearance of the ridges is thus consistent with previous reports from freshwater cable bacteria [7,38].

The amplified 16S rRNA gene fragment (428 bp) of the cultured cable bacterium strain GS was 100% identical to *Ca. Electronema nielsenii* [38]. It accounted for 6.9% and 5.0% of all bacterial reads in two replicate cores of a fully-grown culture, 35 days after inoculation with a sediment clump.

Microsensor measurements confirmed the activity of cable bacteria (Fig. 4). Microprofiling in inoculated single filament transfer

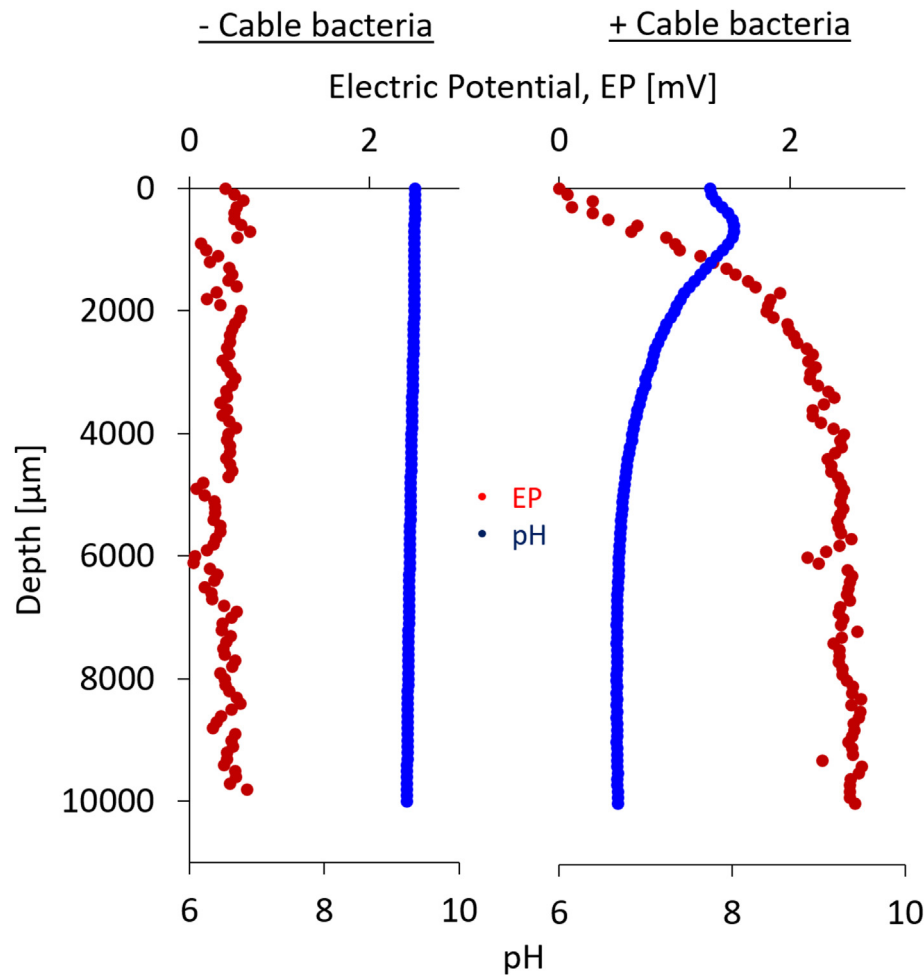


Fig. 4. EP and pH profiles of autoclaved sediment cores without (left) and with (right) addition of a single cable bacteria filament after 19 days of incubation. The inoculated core displays a pH peak (blue) in the upper sediment layer and an electric potential of ~2 mV (red), both indicative of cable bacteria activity. No cable bacteria signature was observed in the un-inoculated core.

cores yielded depth profiles characteristic of cable bacteria growth [30]: a pH minimum in the deeper sediment, a pH maximum at the surface, and an electric field spanning the area in between [10]. Negative control cores showed no change in pH and no EP signal (Fig. 4). There was a clear correlation between the size of the inoculum and the time by which cable bacteria activity was detected (Fig. S2). Cores inoculated with a clump of sediment developed characteristic cable bacteria profiles by day 11, while cores inoculated with a single cable bacterium filament showed lower activity at similar timepoints.

Genome features, phylogeny, and proposal of Candidatus Electronema aureum

A high-quality cable bacterium draft genome was extracted from the combined metagenomes. It has a total size of 2.76 Mbp on 73 contigs, with an estimated genome completeness of 93% and 1.59% contamination. Three single-copy genes occurred twice in the genome, and for each of these duplicates, one copy was a native cable bacterium gene and the other copy originated from outside of the Desulfobulbaceae family. This indicates that the contamination does not result from the co-assembly of two cable bacteria strains and is likely a byproduct of the metagenome binning. The metabolic features of the genome of strain GS are discussed in detail in Kjeldsen et al. [14].

The full-length 16S rRNA gene of strain GS was extracted from the genome assembly and was, in accordance with the amplicon data, almost identical to *Ca. E. nielsenii* (Table 1). However, at the genome level the ANI and AAI to both described freshwater cable bacteria species, *Ca. E. nielsenii* and *Ca. E. palustre* [38], were below the proposed 95% threshold for species delineation [15]. Phylogenetically, the strain GS grouped together with *Ca. E. nielsenii* within the freshwater genus *Ca. Electronema*; this tree topology was well-supported in both the 16S rRNA gene phylogeny (Fig. 5A) and by single-copy phylogenetic marker genes (Fig. 5B). From the combined 16S rRNA, genomic, morphological, and metabolic features we conclude, in accordance with the proposed

Table 1
Comparison of 16S rRNA, ANI and AAI similarities between *Ca. Electronema aureum* and *Ca. Electronema nielsenii* and *Ca. Electronema palustre* (the name has been corrected from “*E. palustris*” in the original publication, according to Oren et al. [24]).

Species name	<i>Ca. Electronema aureum</i> GS		
	16S rRNA	ANI	AAI
<i>Ca. Electronema nielsenii</i> F1	99.36	93.97	92.11
<i>Ca. Electronema nielsenii</i> F5	99.42	93.32	91.08
<i>Ca. Electronema palustre</i> F3	95.36	82.74	76.17
<i>Ca. Electronema palustre</i> F4	95.36	83.08	77.26

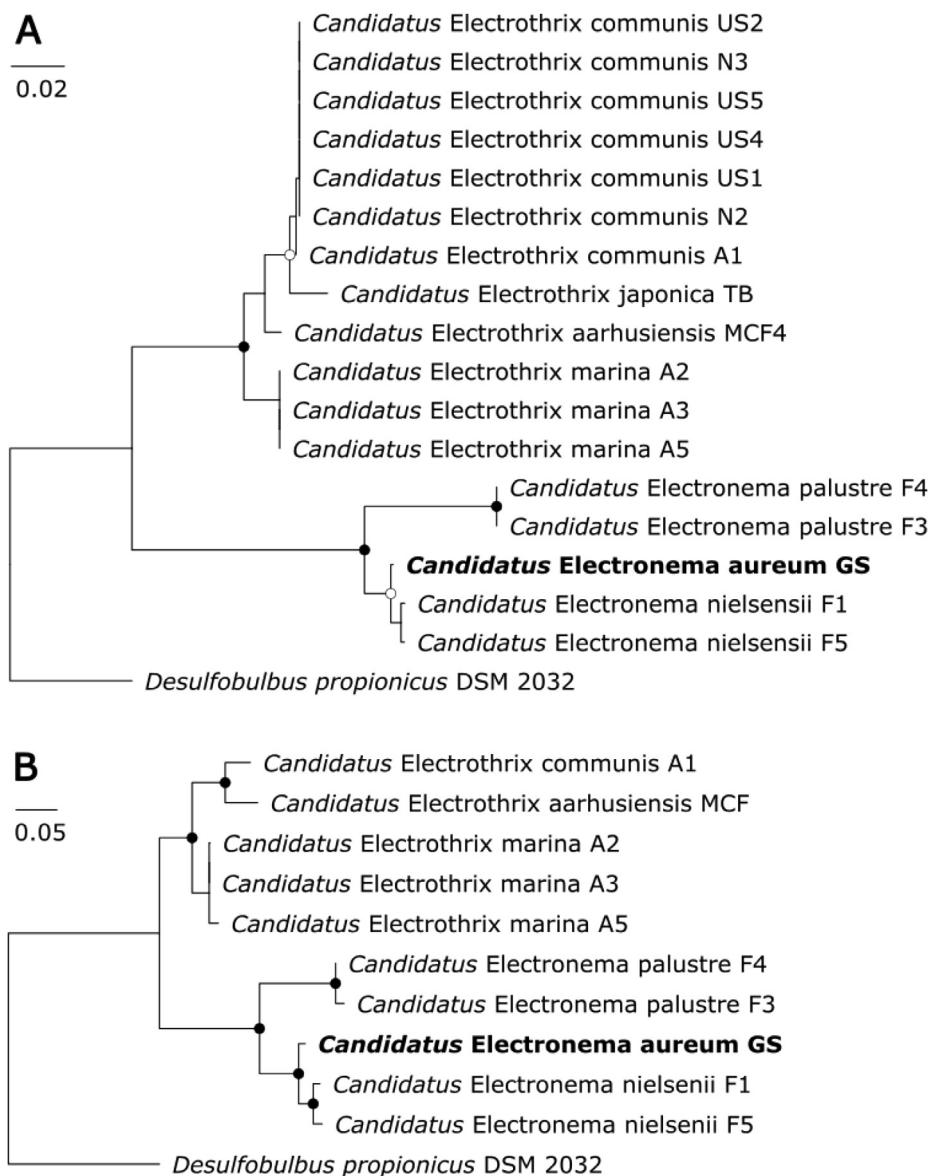


Fig. 5. Phylogenetic affiliation of *Candidatus Electronema* sp. GS (bold) with the two cable bacteria genera *Ca. Electrothrix* and *Ca. Electronema*, based on (A) the 16S rRNA gene and (B) concatenated protein sequences aligned to the GTDB taxonomy. The trees have been rooted with *Desulfobulbus propionicus*. Black circles indicate bootstrap values > 90 and white circles indicate bootstrap values > 70 (n = 1000).

standards for high-quality taxa descriptions of uncultivated Bacteria [15], that strain GS is a new species of cable bacterium, for which we propose the name *Ca. Electronema aureum* GS.

Strain GS: A stable culture facilitating physiological and molecular studies

Our culture of *Ca. E. aureum* GS has by now been stable and active for seven years. Comparing the genome assemblies after the 9th and 11th transfer (approx. 2 months apart) showed an ANI of 99.9%, thus confirming the identity and stability of this cable bacterium strain.

In addition, the cell width of *Ca. E. aureum* GS remained constant over 32 days during a (batch) growth cycle: the median cell width was $1.7 \pm 0.1 \mu\text{m}$ (n = 200/time point) at all time points (Fig. S3), which is well within the error of light microscopy. This strongly suggests that this freshwater cable bacterium does not

change its width over time, as has been observed in saltwater cable bacteria of the genus *Ca. Electrothrix* [32,38].

This stable single-strain culture provides the first reproducible way to monitor and manipulate a specific cable bacterium strain. This will greatly enhance our ability to combine functional and omics-based experiments; a recent study already made use of *Ca. E. aureum* GS for metaproteome identification [14], while another study is currently combining rate measurements and transcriptomics to unravel its nitrogen metabolism [20]. The cultivation of additional cable bacteria species would even allow for future co-cultivation and competition experiments to address the ecophysiology, adaptation, and evolution of cable bacteria.

In conclusion, we have demonstrated the physical isolation and long-term stable growth of a single cable bacterium strain in a partially sterilized sediment system that resembles its natural gradient environment. Similar approaches may be applicable to other difficult-to-culture microbes, especially gradient organisms such

as the large sulfur bacteria *Beggiatoa*, *Thioploca*, or *Thiomargarita* [34].

Description of *Candidatus Electronema aureum*

“*Candidatus Electronema aureum*” au're.um, L. neut. adj. aur-eum, golden, after the whitish-orange crust of oxidized iron and carbonite characterizing the presence of cable bacteria in a sediment culture. Member of the freshwater genus *Ca. Electronema*; cultivated from freshwater pond sediment; filamentous bacteria of centimeter length that conduct electrons from sulfide to oxygen or nitrate. Gliding motility. Gram-negative, with 40 distinct ridges running longitudinally along the filament. Width of individual cells is constant, $1.7 \pm 0.1 \mu\text{m}$. G + C content, 52%. Distinguishable by their morphology and genome; accession number GCA_004284765.1.

Data availability

All sequence data have been submitted to GenBank under the following accession numbers: assembled genome of *Ca. Electronema aureum* GS, Bioproject accession PRJNA389779; metagenome reads, Bioproject accession PRJNA730231; 16S rRNA gene amplicon data, Bioproject accession PRJNA730189. *Ca. Electronema aureum* GS (as sediment culture) is available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.syapm.2021.126236>.

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